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Cyto-ID™ Red Long-Term Cell Tracer Kit

for flow cytometry and fluorescence microscopy

Instruction Manual

Cat. No. ENZ-51037-K025

For research use only.

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Notice to Purchaser

The Cyto-ID™ Red Long-Term Cell Tracer Kit is a member of the CELLestial® product line, reagents and assay kits comprising fluorescent molecular probes that have been extensively benchmarked for live cell analysis applications. CELLestial® reagents and kits are optimal for use in demanding cell analysis applications involving confocal microscopy, flow cytometry, microplate readers and HCS/HTS, where consistency and reproducibility are required.

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I. Introduction

The Cyto-ID™ Red Long-Term Cell Tracer Kit uses proprietary noncovalent cell labeling technology to stably incorporate a red fluorescent dye containing hydrophobic aliphatic chains into the cell membrane's lipid bilayer. The dye may be loaded into cells by following the included protocol. The labeling buffer is isotonic for mammalian cells and contains no detergents or organic solvents. The appearance of labeled cells may vary depending upon the cell type from uniformly bright to punctuate. This difference is thought to relate to the extent of membrane internalization occurring after cell labeling. The Cyto-ID™ Red Tracer Dye fluorescence is independent of pH within normally encountered physiologic ranges and fluorescence intensity per cell is typically unaffected by the ultimate pattern of dye distribution. The Cyto-ID™ Red Tracer Dye is not toxic to cells, as determined using the benchmark MTT cell viability assay. The dye is well retained by cells for up to 96 hours after loading, and is passed to daughter cells upon mitosis. Since the dye does not covalently modify proteins within the cells, normal physiological responses are better preserved than with molecular probes based upon thiol-reactive chloromethyl -based or amine-reactive succinimidyl ester-based fluorescent dyes. Dual labeling is also possible using a variety of available CELLestial® dyes. Labeled cells can be visualized by epifluorescence or confocal fluorescence microscopy. Additionally, dye-labeled and unlabeled cell populations can be analyzed by flow cytometry. No transfer of fluorescence to adjacent cells was observed after a prolonged 96 hour incubation period. This is in stark contrast to Calcein AM and BCECF AM, which are only retained within viable cells for a few hours at physiological temperatures. The kit is suitable for a variety of applications including long term cell viability, cytotoxicity, cell adhesion, cell migration and cell-cell fusion studies.

II. Reagents Provided and Storage

All reagents are shipped on dry ice. Upon receipt, the kit should be stored upright at ≤-20°C, protected from light. When stored properly, these reagents are stable for one year upon receipt. **Avoid repeated freezing and thawing.**

Reagents provided in the kit are sufficient for 25 reactions for flow cytometry or fluorescence microscopy.

Reagent	Quantity
Cyto-ID™ Red Tracer Dye	50 µl
4X Labeling Buffer	12.5 mL
10X HBSS	25 mL

III. Additional Materials That May Be Required

- Flow cytometer equipped with 488nm blue laser
- Fluorescence microscope
- 15 ml and 50 ml conical tubes
- Adjustable speed centrifuge with a swinging bucket rotor
- CO_2 incubator (37°C), tissue culture plasticware, tissue culture reagents
- 5 mL round bottom polystyrene tubes for holding cells
- Calibrated, adjustable precision pipettors, preferably with disposable plastic tips
- Glass microscope slides
- Glass cover slips
- Deionized water
- Serum (*e.g.,* Fetal Bovine Serum)
- Growth medium (*e.g.,* Dulbecco's Modified Eagle Medium, D-MEM)

IV. Safety Warnings and Precautions

- This product is for research use only and is not intended for diagnostic purposes.
- Some components of this kit may contain hazardous substances. Reagents can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. They should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.
- Methods are developed and optimized using kit components. Use only supplied buffers for optimal results.

V. Methods and Procedures

NOTE: *Allow all reagents to thaw at room temperature before starting with the procedures. Upon thawing, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, as well as for all subsequent uses, to gather the contents at the bottom of the tube.*

A. REAGENT PREPARATION

1. 1X HBSS

Allow the 10X HBSS to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution.

For every 10 mL of 1X HBSS needed, dilute 1 mL of 10X HBSS with 9 mL deionized water.

2. Stop Buffer

Prepare the Stop Buffer by adding 200 µL Fetal Bovine Serum (FBS) to 9.8 mL 1X HBSS (from step A1).

3. 1X Labeling Buffer

Dilute each milliliter (mL) of 4X Labeling Buffer with 3 mL deionized water.

4. 2X Cyto-ID™ Red Tracer Dye Solution

IMPORTANT: *Prepare this reagent immediately before labeling cells.*

In an appropriate size container, mix, by vortexing, the following:

2 µL Cyto-ID™ Red Tracer Dye

1mL 1X Labeling Buffer (from step A3)

B. STAINING LIVE, SUSPENSION OR ADHERENT CELLS

NOTE: *Cells are labeled by incorporating the dye into the cellular membrane. Best results are a factor of cell concentration and dye concentration. Loss of membrane integrity and poor cell recovery will result from over staining. Also, best results are obtained when adherent cells are dispersed into a cell suspension prior to staining.*

Perform all subsequent steps at ambient temperature (20-25°C).

1. Place a suspension containing 2 $X10^7$ cells in a 15 mL conical bottom polypropylene tube. Centrifuge cells at 400 x *g* and remove growth medium. Wash twice with 4 mL 1X HBSS.

   NOTE: *Serum proteins and lipids also bind the dye, reducing the effective concentration available for cell labeling.*

2. Centrifuge the cells at 400 x *g* for 5 minutes into a loose pellet. After centrifuging cells, carefully discard the supernatant, being careful not to remove any cells but leaving no more than 25 µL of supernatant.

   IMPORTANT: *For reproducible results, it is important to minimize the amount of residual medium or buffer present when cells are re-suspended in the Labeling Buffer.*

3. Prepare a **2X cell suspension** by adding 1 mL of Labeling Buffer to the cell pellet and suspending with gentle pipetting to insure complete dispersion. **Do not vortex and do not let cells stand in Labeling Buffer for periods longer than 15 - 20 minutes**.

   IMPORTANT: *The presence of physiologic salts like Ca^{+2} and Mg^{+2} causes the dye to form micelles and substantially reduces staining efficiency. Therefore, it is important that the cells be suspended in Labeling Buffer provided at the time dye is added, not in medium or buffered salt solutions.*

4. **Immediately** prior to staining, prepare a 2X Cyto-ID™ Red Tracer Dye Solution in Labeling Buffer as described in step A4 on, p3.

5. Rapidly add the 1 mL of 2X cell suspension (from step B3, above) to 1 mL of freshly prepared 2X Cyto-ID™ RED Tracer Dye solution and **immediately** mix well with a pipetor or equivalent to disperse.

   IMPORTANT: *Staining is nearly instantaneous. Brisk and consistent dispersion of the cells in the Cyto-ID™ Red Tracer Dye solution is vital for consistent labeling.*

6. Incubate the cell-dye suspension from step B4 for 2–5 minutes with periodic mixing. Longer staining periods, not to exceed 7 minutes, will result in brighter cell staining.

   NOTE: *Do not centrifuge the cells in Labeling Buffer before stopping the staining reaction.*

7. Stop the staining by adding an equal volume (2 mL) of Stop Buffer and incubate for 1 minute to allow binding of excess dye. **Do not dilute with Labeling Buffer.**

8. Centrifuge the cells at 400 x *g* for 5 minutes at 25°C and carefully remove the supernatant, being sure not to remove cells. To minimize carryover of residual dye bound to the tube walls, suspend cell pellet in 10 mL of complete (serum-containing) medium, transfer to a fresh sterile conical polypropylene tube and centrifuge at 400 X *g* for 5 minutes at 25°C, and wash the cell pellet 2 more times with 10 mL of complete medium each wash to ensure removal of unbound dye.

   NOTE: *Staining efficiency is increased by transferring into a fresh tube at the first suspension step after staining. Do not use Labeling Buffer for washing steps.*

9. After the final wash, suspend the cell pellet in 10 mL of complete medium and transfer to a T25 flask or slides and incubate at 37°C and 5% CO_2 overnight for no less than 12 hours, to allow cells to recover.
10. Perform cell counting and viability assessment. After determining cellular recovery and viability, the following steps may be performed:
    a. Centrifuge the cell suspension and suspend to desired concentration of viable cells.
    b. Change the medium of the adherent cells.

C. Cell Analysis By Fluorescence/Confocal Microscopy

Suspension Cells

1. Collect the stained cells by centrifugation for 5 minutes at room temperature at 400 x *g and c*arefully discard the supernatant . Re-suspend the cells with at least 20 µL of 1X HBSS.
2. Apply 20 µL of the cell suspension to a glass microscope slide and overlay with a coverslip.
3. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard Texas Red filter set for imaging the membrane signal.

Adherent Cells

1. Seed stained cells at your desired density (such as 10^4/ mL). Allow to adhere overnight. Remove growth medium add 1X HBSS as needed to maintain cell moisture and overlay with a coverslip.
2. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard Texas Red filter set for imaging the membrane signal.

D. Cell Analysis By Flow Cytometry

The protocol described in this manual assumes that the user is familiar with the basic principles and practices of flow cytometry and is able to run samples according to the operator's manual pertaining to the instrument being used.

1. Collect stained cells at a density not to exceed 1X 10^6 cells/mL.

   NOTE: *Adherent cells will need to be trypsinized prior to analysis on the flow cytometer.*

2. Centrifuge at 400 x *g* for 5 minutes to pellet the cells. Carefully suspend the cells in 500 µL of 1X HBSS
3. Read on bench top flow cytometer by gating out cellular debris and using a 488 nm blue laser for excitation and FL2 or FL3 for signal registration.

VI. APPENDICES

A. Filter Set Selection

The selection of optimal filter sets for a fluorescence microscopy applications requires matching the optical filter specifications to the spectral characteristics of the dyes employed in the analysis. Consult the microscope or filter set manufacturer for assistance in selecting optimal filter sets for your microscope. Use a standard Texas Red filter set for imaging the membrane signal.

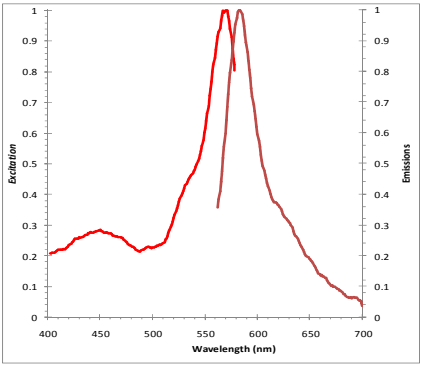


Figure 1. Fluorescence excitation (Ex_{max} 450nm, 570nm) and emission (Em_{max} 583nm) spectra for the Cyto-ID™ Red Tracer Dye. All spectra were determined for cell-bound dye.

B. Expected Results

Labeled cells can be visualized by epifluorescence microscopy using a standard Texas Red filter (Figure 2).

Dye-labeled and unlabeled cell populations can be analyzed by flow cytometry. No transfer of fluorescence to adjacent cells was observed after a prolonged 96 hour incubation period (Figure 3).

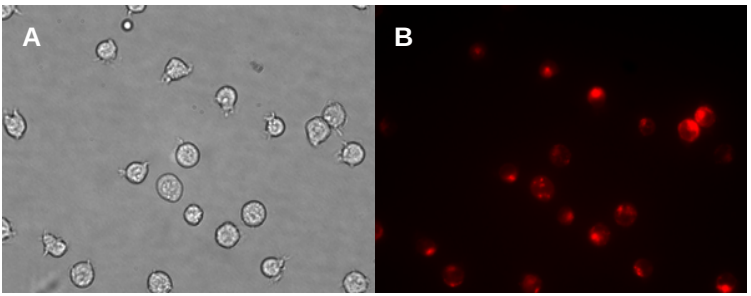
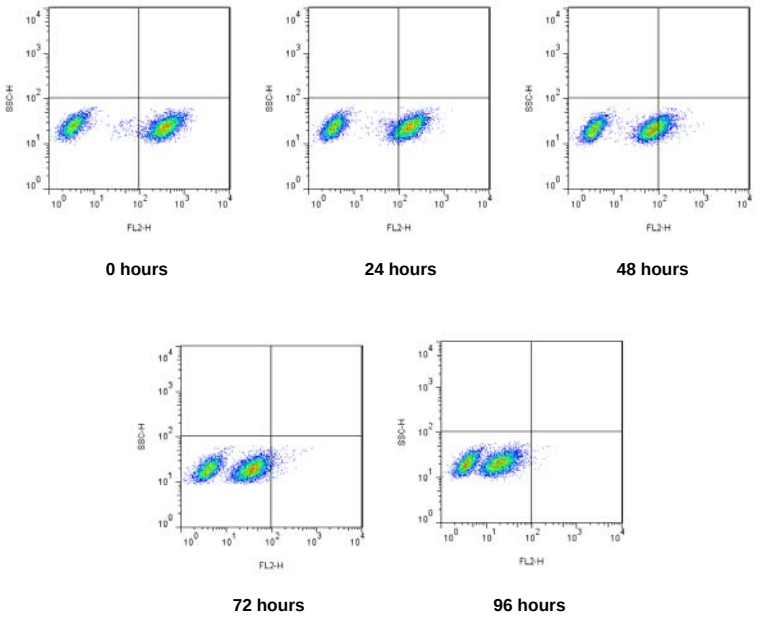


Figure 2: Composite bright-field (panel A) and fluorescence microscopy (panel B) images demonstrating staining of Jurkat cells with Cyto-ID™ Red Tracer Dye. Standard Texas Red filter set was used to image the membrane-bound signal.



Time Post-Mixing	% of Stained Cells	MFI Stained Cells	% Unstained Cells	MFI Unstained Cells
0 Hour	61	405	36	3.7
24 Hour	61	185	37	3.8
48 Hour	62	86	36	3.9
72 Hour	62	42	36	4.2
96 Hour	61	22	37	3.9

Figure 3: Flow Cytometry analysis of fluorescence of mixed population of Jurkat cells over time. Jurkat cells stained with Cyto-ID™ Red Tracer Dye were mixed with an unstained population of Jurkat cells and incubated over a 96 hour period.

VII. References

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2. Horan, P.K. et al. Methods Cell Biol. **33**:469-490(1990).
3. Poon, R.Y. et al., in: In Living Color: Flow Cytometry andCell Sorting Protocols, Diamond, R. A., and DeMaggio,S., (eds.), Springer-Verlag, (New York, NY: 2000) pp.302-352.
4. Wallace, P.K., and Muirhead, K.A., Immunol. Invest., **36**:527-562 (2007).

VIII. Troubleshooting Guide

Problem	Potential Cause	Suggestion
Poor viability or recovery of cells.	Too much dye was incorporated into the cell membranes.	Lower dye concentration and/or increase cell number.
	Cells remained in the labeling buffer too long.	Decrease the time that cells are in the labeling buffer. The maximum time is 15-20 minutes.
Cross-staining of cell populations	Labeled cells lysed, releasing dye to adjacent cells.	Lower dye concentration and/or increase cell number.
	Excess dye in solution or on the walls of the tube.	Wash cells 3-5 times after labeling. Transfer the samples to new tubes between washes. Be careful to remove as much liquid as possible during wash steps.
	Organic based solvents or detergents were used that may extract the dye.	Use only aqueous-based fixatives and solvents and limit the use of detergents.

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